FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR
216324US0PCT	09/926622

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/CA00/00621	25 MAY 2000	28 MAY 1999

TITLE OF INVENTION

INACTIVATION OF FOOD SPOILAGE AND PATHOGENIC MICROORGANISMS BY DYNAMIC HIGH PRESSURE

APPLICANT(S) FOR DO/EO/US

Paul PAQUIN, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Request for Consideration of Documents in International Search Report
Notice of Priority / PCT/IB/308

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/926622	PCT/CA00/00621	216324US0PCT

24. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	$1040.00
☒	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	$890.00
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	$740.00
☐	International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)	$710.00
☐	International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)	$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT = $890.00

Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)). $130.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	7 - 20 =	0	x $18.00	$0.00
Independent claims	1 - 3 =	0	x $84.00	$0.00
Multiple Dependent Claims **(check if applicable)**.			☐	$0.00

TOTAL OF ABOVE CALCULATIONS = $1,020.00

☐ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2. $0.00

SUBTOTAL = $1,020.00

Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). + $0.00

TOTAL NATIONAL FEE = $1,020.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐ $0.00

TOTAL FEES ENCLOSED = $1,020.00

Amount to be: refunded	$
charged	$

a. ☒ A check in the amount of **$1,020.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **15-0030** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

22850

Surinder Sachar
Registration No. 34,423

(703) 413-3000

Surinder Sachar
SIGNATURE

Norman F. Oblon
NAME

24,618
REGISTRATION NUMBER

11-27-01
DATE

INACTIVATION OF FOOD SPOILAGE AND PATHOGENIC MICROORGANISMS BY DYNAMIC HIGH PRESSURE

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to a process for inactivation of contaminating liquid food pathogens, and more particularly to such a process which utilize a dynamic high-pressure treatment.

(b) Description of Prior Art

Every year, outbreaks of illnesses caused by pathogenic bacteria contaminating foods have economic repercussions throughout the world. Due to its composition and mode of production, milk is particularly susceptible to contamination by a wide variety of bacteria. When milk is secreted in the udders of ruminants, it is virtually sterile. Many milk-borne bacteria are casual visitors but find them in an environment where they can live and possibly proliferate. Although some of these bacteria die when competing with species which find the environment more congenial pathogenic bacteria, such as *Listeria, Escherichia, Salmonella,* can survive and create dangers for the consumer.

Heat, for instance pasteurization is still the most commonly used technology to inactivate food spoilage and pathogenic bacteria in raw milk and other liquid foods. Although effective, some bacteria may resist thermal treatment, especially Bacillus and Clostridium. Furthermore, high temperatures may induce undesirable losses of flavor as well as denaturation of certain vitamins and nutritive proteins. Reduction in soluble calcium, formations of complexes between constituents, and reduction of cheese yield have also been observed. For example, thermal decomposition of milk β-lactoglobulin produces volatile sulfur compounds

that may inhibit fermentation, thus affecting the appearance, taste and nutritional value of milk as well as processing characteristics.

In recent years, many alternative methods have been investigated as means of inactivating food spoilage and pathogenic bacteria. Bactofugation and microfiltration have been proposed and shown to reduce the initial microbial load. These processes still required a heat treatment in order to achieve satisfactory results. The advantages of these methods are better microbial quality and longer shelf life. More recently, high hydrostatic pressure (HHP) technology has been proposed as a new strategy to inactivate both the spoilage and pathogenic bacteria. Using this technology, high pressure (5 to 15 kbars or 500 to 1500 MPascal (MPa)) are often needed to achieve the inactivation effect. Such pressures may affect systems determining morphology, biochemical reactions, genetic mechanisms, membrane, and cell wall structure of microorganisms. Sensivity to high pressure varies greatly from one bacterial specy to another. A pressure of 300 MPa (3000 bars) for 10 to 30 minutes is needed for the inactivation of Gram positive bacteria, yeasts and mildew. *Bacillus subtilis* spores are inactivated at 1750 MPa. A pressure of 400 MPa for 20 minutes is required to completely inactivate *E. coli* or bring about an 8-log reduction of *Saccharomyces cerevisiae.* Unfortunately, the principle of this technology is applied as a batch treatment, that is suitable for small volumes, and the establishment of this method on an industrial scale is difficult and costly.

It is well known that ultraviolet light in the proper dose kills most bacteria, algae, viruses, mold spores, and other microorganisms found in liquids such

as water. There have been many ultraviolet water sterilization systems proposed to take advantage of this phenomenon. U.S. Pat. Nos. 4,769,131 and 4,968,437 issued to Noll et al. disclose an ultraviolet purification system in which water is pumped through tubes helically coiled around an ultraviolet lamp to provide maximum ultraviolet exposure time for a given tube length to create a relatively compact sterilization system for potable water.

This system as well as other known systems suffers from a number of drawbacks which make them less than ideal solutions to the water purification problem. Ultraviolet sterilization is not applicable on milk because of the opalescence.

On problem common to these systems is that the liquid must be pumped under pressure past the ultraviolet lamp both before and after filtration. This requires a relatively large pump that draws a relatively great amount of power. In addition, such systems are typically designed to treat tap water, and are incapable of taking water from another source such as collecting water dripping off a condensing coil of a dehumidification or air conditioning system.

In the sterilization of milk, it is necessary to raise the temperature of the milk sufficiently to destroy all bacteria and inactivate enzymes. The rate of destruction or inactivation of these organisms varies with both temperature and the time during which the product is held at an elevated temperature. A method of sterilizing milk and dairy products has been to utilize steam infusion to subject the milk to ultra high temperatures for very short periods of time followed by flash cooling. This has been proven to achieve superior product flavor. Various approaches have been used in the past to accomplish this. For

example U.S. Pat. No. 3,156,176 to Wakeman describes a heating apparatus in which steam is supplied into a chamber with the liquid product being introduced in the form of a curtain-like film to expose the fluent product to the elevated steam temperatures. Similarly, U.S. Pat. No. 2,899,320 to Davies and U.S. Pat. No. 3,032,423 to Evans, both utilize apparatus for containing steam in which the product is passed over plates within the steam chamber and heated while the product flows downwardly to a collection point for delivery to a flash chamber. A variation of this method is also described in U.S. Pat. No. 3,771,434 to Davies in which screen panels are used to form a thin film of product for exposure to steam. One major disadvantage of the methods and apparatus described in the foregoing patents is the fact that liquid food products, particularly milk products, have a tendency to burn and collect on heated surfaces which are at temperatures greater than or equal to the temperature of the product itself. Such burning, in addition to fouling the apparatus itself necessitating periodic cleaning, also results in undesirable flavor changes to the milk product.

In an obvious effort to avoid such burn-on and fouling, U.S. Pat. No. 4,310,476 to Nahra and U.S. Pat. No. 4,375,185 to Mencacci attempt to form free falling thin films of milk within a steam atmosphere for raising the product temperature. A problem associated with attempting to form a free falling thin film is that the integrity of such films is very unstable and are subject to splashing or break-up in the presence of moving or circulating steam and gases. Film formation requires close adherence to flow parameters and such devices are also subject to the product burn-on problems when hot surfaces are contacted. Additionally,

it is recognized as discussed in the Nahra patent that physical agitation of milk may also affect the ultimate flavor of the treated product and disturbance of the free falling films will result in such agitation.

US Patent 6,019,947 discloses a method and apparatus for sterilization of a continuous flow of liquid, which utilize hydrodynamic cavitation. This apparatus uses relatively low pressure (200 to 500 PSI), and the only one cellular lytic mechanism is cavitation. The maximum sterilization yield allows reduction in bacterial counts of only 4 logs.

US Patent 5,232,726 discloses a method for reducing the microbial activity in juices by continuous high-pressure homogenization of citric juices. While results in applying this method are highly variable and inconsistent, lower pressure seems to give as much good effects than higher pressure. The maximum pressure of 15 000 psi has been used in this method, which is considered as a low pressure for those well skilled in the art.

Another problem associated with many of the prior art approaches to steam infusion of liquid products is that the devices are not easily cleaned for example with the use of clean-in-place systems. The more internal components in which the product may collect or burn-on, the more difficult the cleaning process.

It would be highly desirable to be provided with a new process allowing pasteurization of liquid food products without affecting the nutritive value, and preserving all other characteristics of the liquid, like flavor.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide a process for continuously reducing presence of microorganisms in liquid food product without denaturation consisting of: a) pressurizing a liquid food product; b) passing a liquid food product to be treated through a

continuous pressurizing circulating system at a non-denaturing temperature comprising a dynamic high pressure homogenizer; and c) collecting the liquid food product containing a reduced presence of microbes.

Another aim of the present invention is to provide a process wherein the pressure used is between 50 MPa to 500 MPa.

In accordance with the present invention there is provided also a process that needs at least one passage of the liquid food product through the dynamic high-pressure homogenizer.

Another aim of the present invention is to provide a process wherein the microorganisms to be killed may be selected from bacteria, fungi, mould, bacteriophage, protozoan, and virus.

The process may be performed using a milk homogenizer at temperature between 4°C to 55°C.

Also, one aim of the invention is to provide a process of sterilizing several liquid food products as of milk, juice, liquid food fat, oil, and water.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the inactivation of three major food pathogens in phosphate buffer by DHP as a function of applied pressure (100, 200 and 300 MPa) and the number of passes (1, 3 and 5).

Fig. 2 illustrates the inactivation of *Listeria monocytogeneses* (■), *Salmonella enteritidis* (■), *Escherichia coli* (□) in phosphate buffer by DHP (200 MPa/1 pass) after a mild heat treatment for 10 minutes at 4, 25, 45 or 55 °C.

Fig. 3 illustrates the inactivation of *Listeria monocytogeneses* (■), *Salmonella enteritidis* (■) and *Escherichia coli* (□) in phosphate buffer by DHP (200 MPa/1 pass) as a function of initial bacterial load (10^4 to 10^9).

Fig. 4 illustrates the inactivation of two major food pathogens in raw milk by DHP as a function of applied pressure (100, 200 and 300 MPa) and number of passes (1, 3 and 5).

Fig. 5 illustrates the inactivation of two major food pathogens in raw milk by DHP (200 MPa/1 pass) in response to a mild heat treatment of 10 minutes (25, 45, 55 and 60 °C).

Fig. 6 illustrates the inactivation of two major food pathogens in raw milk by DHP (200 MPa/1 pass) as a function of initial load (10^5 to 10^8).

Fig. 7 illustrates the inactivation of *Listeria innocua* (10^7 CFU/ml) in raw milk by DHP (200 MPa) at a laboratory (Emulsiflex-C5) or industrial scale (Emilsiflex-C160).

DETAILED DESCRIPTION OF THE INVENTION

The use of dynamic high-pressure to inactivate food pathogens has never been reported. In contrast to hydrostatic high-pressure treatment (HHP), the dynamic high pressure (DHP) uses low pressure, as about 2 kbars to achieve same bacteria inactivation results. At this relatively low pressure, food constituents are better preserved from mechanical and biophysical damages well characterized in other sterilization approaches.

In accordance with the present invention, there is provided an new alternative to liquid food pasteurization, that is to say dynamic high pressure (DHP). In the milk industry, light pressure homogenization is used to reduce the diameter of fat globules in order to prevent creaming. Pressure is applied to a liquid forced through an adjustable valve causing increased flow speed and a pressure loss, bringing about cavitation, chisel effect, turbulence

and collision on the stationary surface, which combine to reduce the size of fat globules.

In a preferred embodiment of the invention, microorganisms are disrupted by a multiplicity of mechanisms during submitting to DHP: the sudden pressure drop, shear stresses, cavitation and impingement. The overall pressure drop and the rate at which it occurs can is responsible for the cell disruption.

It will be apparent to those skilled in the field that the method and apparatus thus described is extremely simple, avoids the problem of product burn-on.

In a particular embodiment of the invention, there is provided with a process to treated liquid food products contaminated, or potentially contaminated with, but not limitatively, Gram positive or Gram negative bacteria, yeast, viruses, protozoan, and mould.

In one embodiment of the invention is to preformed sterilization to pressure up to 40 000 psi(277 Mpa).

In accordance with another embodiment of the invention, the DHP can be applied in inactivating bacteriophages in different liquid food products, or also to inactivate enteric viruses such as Hepatitis A, rotavirus, and Norwalk virus contained in water.

It is recognized from the present invention that several food products lend themselves to preservation by the use of DHP to sterilize the products. DHP sterilization destroys microorganisms and inactivates most enzymes that cause product spoilage.

One embodiment of the invention as extending normal shelf life of fresh food while at same time maintaining nutritional quality and ensuring safety, as for example milk, and cheese.

Also, the invention relates to a process for eliminating lactic acid bacteria bacteriophages from cheese plant by treating milk and whey samples.

An another embodiment of the invention is that DHP sterilization of certain food products may eliminate the need for refrigeration. This is particularly true in the case of dairy products such as milk or ice cream mix, to which this invention is primarily directed, although it may be equally applied to other liquid products such as juices.

While the invention has thus been described in relation to a process for treating milk, others skilled in the art will appreciate that other food products in liquid form may also be sterilized as well such as flavored milk, half and half, dairy creams, whipping creams, condensed milk, ice cream milk, shake mix, puddings, custard, fruit juices, etc. Adjustments to the operating pressure and flow rates may be necessary but these variations will be recognized and easily addressed by those skilled in the field.

EXAMPLE 1

INACTIVATION OF SOME FOOD PATHOGENS USING DYNAMIC HIGH PRESSURE

Every year, outbreaks of illnesses caused by pathogenic bacteria contaminating foods have economic repercussions throughout the world. Due to its composition and mode of production, milk is particularly susceptible to contamination by a wide variety of bacteria. When milk is secreted in the udders of ruminents, it is virtually sterile. Many milk-borne bacteria are casual visitors but find themselves in an environment where they can live and

possibly proliferate. Although some of these bacteria die when competing with species which find the environment more congenial pathogenic bacteria such as *Listeria, Escherichia, Salmonella,* etc, can survive in milk and create dangers for the consumer.

Heat (e.g. pasteurisation) for instance pasteurisation is still the most commonly used technology to inactivate food spoilage and pathogenic bacteria in raw milk. Although effective, some bacteria may resist thermal treatment, especially *Bacillus* and *Clostridium.* Furthermore, high temperatures may induce undesirable losses of flavours as well as denaturation of certain vitamins and proteins. Reduction in soluble calcium, formation of complexes between β-lactoglobulin and κ-casein and reduction of cottage cheese yield have also been reported. Thermal decomposition of β-lactoglobulin produces volatile sulfur compounds (Desmazeaud, 1990) which may inhibit lactic fermentation, thus affecting the appearance, taste and nutritional value of milk as well as its processing characteristics.

In recent years, many alternative methods have been investigated as means of inactivating food spoilage and pathogenic bacteria. Bactofugation and microfiltration shows to reduce the initial microbial load. These processes still required a heat treatment in order to achieve satisfactory results. The advantages of these methods were better microbial quality and longer shelf life. Recently, high hydrostatic pressure (HHP) technology has been proposed as a new strategy to inactivate both the spoilage and pathogenic bacteria. Using this technology, high pressures (1 to 15 kbars or 100 to 1 500 MPa) are often

needed to achieve the inactivation effect. Such pressures may affect systems determining morphology, biochemical reactions, genetic mechanisms, membrane and cell wall structure of microorganisms. Sensitivity to high pressure varies greatly from one bacterial species to another. A pressure of 300 MPa (3 000 bars) for 10 to 30 minutes is needed for the inactivation of Gram negative bacteria, yeasts and mildew. *Bacillus subtilis* spores are inactivated at 1 750 MPa (17 500 bars). A pressure of 400 MPa for 20 minutes is required to completely inactivate *E. coli* or bring about an 8-log reduction of *Saccharomyces cerevisiae*. Furthermore, 500 MPa at 25°C for 20 minutes is required to completely inactivate *Listeria innocua*. The principle of this technology is applied as a batch treatment, which is suitable for small volumes but the establishment of this method on an industrial scale is difficult and costly.

Another alternative to heat is dynamic high pressure (DHP). In the milk industry, light pressure homogenization is used to reduce the diameter of fat globules in order to prevent creaming. Pressure is applied to a liquid forced through an adjustable valve causing increased flow speed and a pressure loss, bringing about cavitation, chisel effect, turbulence and collision on the stationary surface, which combine to reduce the size of fat globules. The effects of DHP on bacterial cells are not yet well known. Some studies have shown changes in cell morphology as well as splits in the cytoplasmic membrane. Decreased numbers of ribosomes and the formation of spongy clear areas within the cytoplasm have also been observed. Research has shown that the cellular membrane is the

site most damaged by pressure. Made of phospholipids and proteins held together by hydrogen bonds ties and hydrophobic bonds, the membrane is somewhat rigid and plays a significant role in cellular respiration and transport. Increases in permeability or rupture of the cell membrane, as may happen under pressure, cause cell death. Based on this principle, DHP technology may offer a promising alternative for the cold pasteurization of milk and perhaps other liquid foods by inactivating bacterial contaminants. A more effective inactivation may be achieved using DHP compared to HHP.

The objective of this study is to evaluate the effectiveness of a dynamic high-pressure treatment for the inactivation of three major food pathogens *Listeria monocytogeneses, Salmonella enteritidis* and *Escherichia coli O157:H7* in raw milk.

Material and methods

Sample preparation: Three bacterial strains were used in this study: as *Listeria monocytogenese* (Canadian Food Inspection Agency #105-1) as Gram positive and *Escherichia coli* O157:H7 (ATCC #35150) and *Salmonella enteritidis* (ATCC #13047) as Gram negative representatives. Bacterial strains were maintained as glycerol stock at -80°C. When needed, strains were inoculated in tryptic soy broth (Difco) and incubated at 37°C for 12 to 18 hours. The culture was then centrifuged at 7 000 rpm for 15 minutes, washed 2 times in phosphate buffer and then used to inoculate different samples of raw milk and phosphate buffer. The final bacterial concentration was determined by enumeration on tryptic soy agar (Difco). The

efficiency of the DHP treatment was estimated by the enumeration of residual bacteria in the sample and was expressed as N/N_o when N_o is the bacterial count before the DHP treatment and N, the residual bacterial count.

DHP treatment of phosphate buffer

Dynamic high pressure was performed using an Emulsiflex-C5 homogenizer (Avestin, Ottawa). Parameters tested were pressure (100, 200 and 300 MPa) and number of passes (1, 3 and 5). We also tested the combined effect of a 10 minute heat treatment at 25, 45, 55 or 60 °C before DHP treatment at 200 MPa for one pass and the effect of initial bacterial concentration on the DHP treatment (200 MPa /1 pass). 50 ml of phosphate buffer (pH 7.3) was inoculated at a concentration of 10^8-10^9 CFU/ml. The sample was then treated at dynamic high pressure under different conditions. An enumeration for each bacterial strain was made on TSA (Difco) to determine the number of CFU for each treated sample. A serial dilution was made in phosphate buffer and 20 µL was plated on TSA. The phosphate buffer samples were observed by electron microscopy for each treatment (100, 200 and 300 MPa) to visualise the effect of high pressure on bacterial cells.

DHP treatment of raw milk

Fresh raw milk was obtained from Natrel (Quebec city, Can.) the day of the experiment and divided into 50-ml samples. Each sample was then inoculated with different concentrations of *L. monocytogeneses* or *E. coli* and submitted to a DHP treatment as described above. Residual bacteria were enumerated on selective medium. Oxford medium base use with Bacto Modified Oxford Antimicrobic Supplement (Difco) was used for

enumerating *L. monocytogeneses* and MacConkey Sorbitol Agar (Difco) was used for *E. coli*. Results were expressed as N/N_0.

Industrial trial

A pilot-scale test was performed at Avestin Inc. in Ottawa to evaluate the efficiency of the industrial device. Dynamic high-pressure was performed using an Emulsiflex-C160 homogenizer (Avestin, Ottawa) with a flow rate of 160 L/h. For this purpose, a raw milk sample (800 ml) was inoculated with *L. innocua* at 10^7 CFU/ml and submitted to a DHP treatment at a pressure of 200 MPa with 1, 3 and 5 passes. The efficiency of the treatment applied was evaluated by enumerating the residual *L. innocua* in modified Oxford medium and by calculating the N/No ratio. Results were compared to those obtained in the laboratory using the Emulsiflex-C5.

RESULTS

Phosphate buffer results: Fig. 1 illustrates the effect of dynamic high pressure treatment at different pressure (100, 200 and 300 MPa) on three different strains (Panel A : *Salmonella enteritidis;* Panel B : *Listeria monocytogeneses;* Panel C : *Escherichia coli*. ■ :1 pass; ■ :3 passes; □ :5 passes; □ :HHP). In general, Gram (+) bacteria (*L. monocytogeneses*) are more resistant to high pressure than Gram (-) bacteria. For *L. monocytogeneses*, a DHP of 300 MPa with 3 successive passes was needed to achieve a total reduction (8 log), compared to *E. coli* or *S. enteritidis* that were completely inhibited at 200 MPa after 3 passes. The resistance of *L. monocytogeneses* to DHP is probably due to its wall-structure, which is made up of a large number of peptidoglycan layers.

This wall composition imparts to the cell a higher resistance to physical phenomena such as chisel effect, turbulence and cavitation undergone by cells in the homogenizer chamber. Gram (-) cells do not have this characteristic and are less resistant. Most of the dead bacteria show a rupture of the cell envelope due to the DHP treatment. For other bacteria, death resulted from total release of the intracellular material without the rupture of the cell envelope.

Previous research on HHP has shown that pressures between 450-500 MPa lasting 10 to 15 minutes are necessary to obtain a reduction of 7 to 8 log units for *L. innocua* (Gervilla, 1997). Rosella Liberti used 600 MPa of static pressure for 10 minutes to get a 5 log reduction from 10^7 to 10^2 CFU/ml with *L. monocytogeneses*. Similar results with *L. monocytogeneses* were obtained after 3 passes under a pressure of 300 MPa in dynamic pressure. DHP was thus more effective than HHP.

Generally, we observe that the more pressure increases, the higher is the death rate. This fact is more evident in panel B with *L. monocytogeneses*. At 100 MPa, the death rate is very low to compared with 300 MPa. The pressure required to eliminate bacteria depends on temperature, pH, chemical composition of the sample and other factors. The number of passes is also a major factor affecting bacterial concentration.

The effectiveness of DHP appears to be affected by the initial temperature of the sample (Fig. 2). An increase in sample temperature prior to DHP treatment results in a better inactivation rate especially for *Salmonella* and *Listeria*. However, no such effect was observed with *E. coli*. For *Salmonella*, heating the

sample to 55°C for 10 minutes results in an additional 4 log reduction after DHP treatment. Two and one additional log reductions were also obtained for 45°C and 25°C respectively. For *Listeria*, only 1.5 additional log reduction was obtained when the sample was heated to 55°C for 10 minutes prior to DHP treatment compared to unheated samples. Heat likely weakens the cell membrane hydrogen and hydrophobic bonds and the bacteria consequently become less resistant to high pressure.

The impact of initial load on the DHP treatment (200 MPa/1pass) is shown in Fig. 3. In general, best inactivation rates were obtained with the lowest bacterial concentration. Once again, *L. monocytogenes* was shown to be the more resistant bacteria compared to the other strains. For *Listeria*, a total inactivation effect was obtained at a concentration of 10^4 CFU/ml while the same effect was obtained at 10^6 and 10^7 CFU/ml for *S. enteritidis* and *E. coli* respectively.

Raw milk results: Two pathogens were tested in milk samples, *L. monocytogenese* and *E. coli*. The effect of pressure and number of passes is shown in Fig. 4 (Panel A : *Listeria monocytogeneses;* Panel B : *Escherichia coli.* ■ :1 pass; ■ :3 passes; □ :5 passes). The reduction of viable bacteria is generally a little more then 2 log smaller than that obtained in phosphate buffer experiments. At 200 MPa (5 passes), a 5.3 log reduction was obtained in the phosphate buffer, whereas in raw milk, only 2.6 reduction was obtained for *L. monocytogeneses*. This phenomenon is even more evident under 300 MPa pressure with 8.3 log and 5.6 log for phosphate buffer and milk respectively.

This difference can be related to the fact that some milk elements such as proteins and fat should have a protective effect on bacteria. The bacteria were fixed to the fat globules and when the sample was homogenized, these globules reduce the effect of physical phenomena such as cavitation, chisel effect and turbulence on the bacteria. This effect was less evident at low pressures. Starting with a microbial concentration of 10^8 CFU/ml, a drop of 1 log was observed even after 5 passes for both the buffer and milk with *L. monocytogeneses*.

The effect of mild heat treatment before homogenization on bacterial reduction in a sample of milk is shown in Fig. 5 (■ *Escherichia coli;* ■: *Listeria monocytogeneses*). The tested temperatures were 25, 45, 55 and 60°C and the pressure was maintained at 200 MPa for only one pass. We observed that the effect was minor at the lower temperatures (25 and 45 °C) but considerable at the higher temperatures (55 and 60°C). With heating at 60 °C, we obtained a difference of 1.1 log for *E. coli* and 1.5 log for *L. monocytogenese* compared to 55 °C which we attribute to the same membrane effects as in phosphate buffer.

The impact of initial load on the DHP treatment (200 MPa/1pass) milk is shown in Fig. 6. (■ *Escherichia coli;* ■ *:Listeria monocytogeneses*). Contrary to the buffer result, we noted no effects on bacterial viability. We explain this result by the protective effect of milk. For each concentration, the effect is the same on the bacteria. This may be due to fat globules binding to the bacteria and protecting them.

Finally, Fig. 7 shows the industrial trial compared to laboratory results for *Listeria innocua* under the same treatment conditions as above. A similar reduction was obtained (□:1 pass; ■ :3 passes; ■ :5 passes).

This study has shown the effectiveness of DHP for destroying pathogenic flora in milk. It has been shown to be a viable alternative to conventional milk pasteurisation. A better bactericidal effect was obtained compared to hydrostatic pressure and milk characteristics were not affected. This new technology should be given serious consideration in the milk industry.

The embodiment(s) of the invention described above is(are) intended to be exemplary only. The scope of the invention is therefore intended to be limited solely by the scope of the appended claims.

ART 34 AMDT

WHAT IS CLAIMED IS:

1. A process for continuously reducing presence of microorganisms in liquid food product without denaturation comprising the steps of:

a) pressurizing a liquid food product;

b) passing said liquid food product to be treated at least two times through a continuous pressurizing circulating system at a non-denaturing temperature comprising a dynamic high pressure homogenizer; and

c) collecting said liquid food product containing a reduced presence of microbes.

2. The process according to claim 1, wherein said pressure of step a) is between about 50 MPa to 500 MPa.

3. The process according to claim 1, wherein said passage of step b) is at least one passage of said liquid food product through the dynamic high pressure homogenizer.

4. The process according to claim 1, wherein said microorganisms are selected from the group consisting of bacteria, fungi, mould, bacteriophage, protozoan, and virus.

5. The process according to claim 1, wherein said temperature is between about 4°C to 55°C.

6. The process according to claim 1, wherein said homogenizer is a high-pressure homogenizer.

7. The process according to claim 1, wherein said liquid food product is selected from the group consisting of milk, juice, liquid food fat, oil, and water.

 AMENDED SHEET

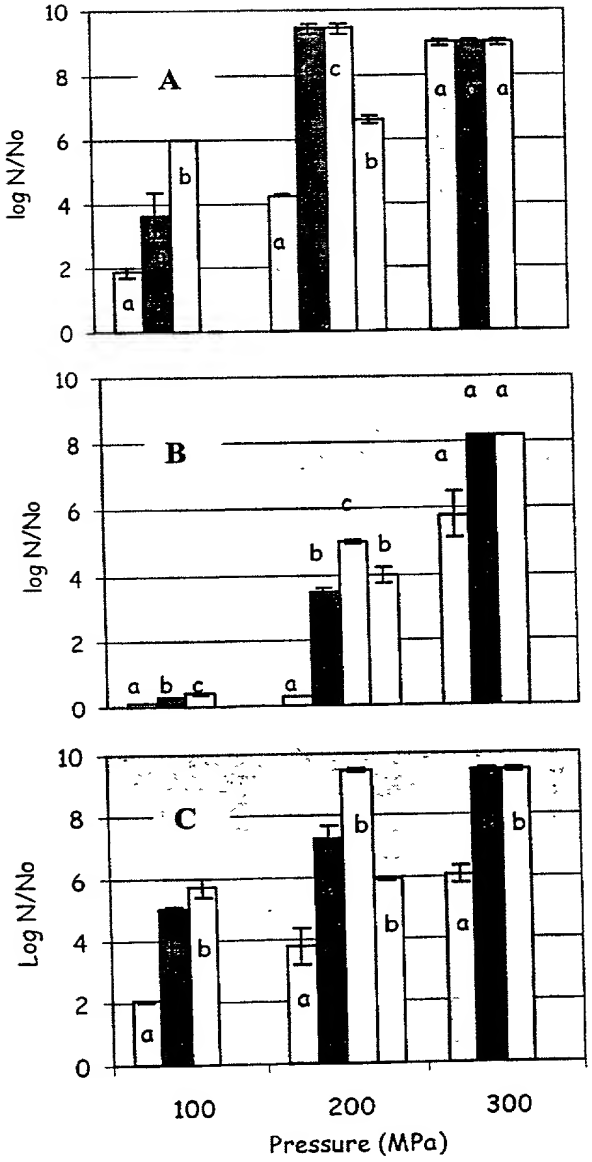


Fig. 1

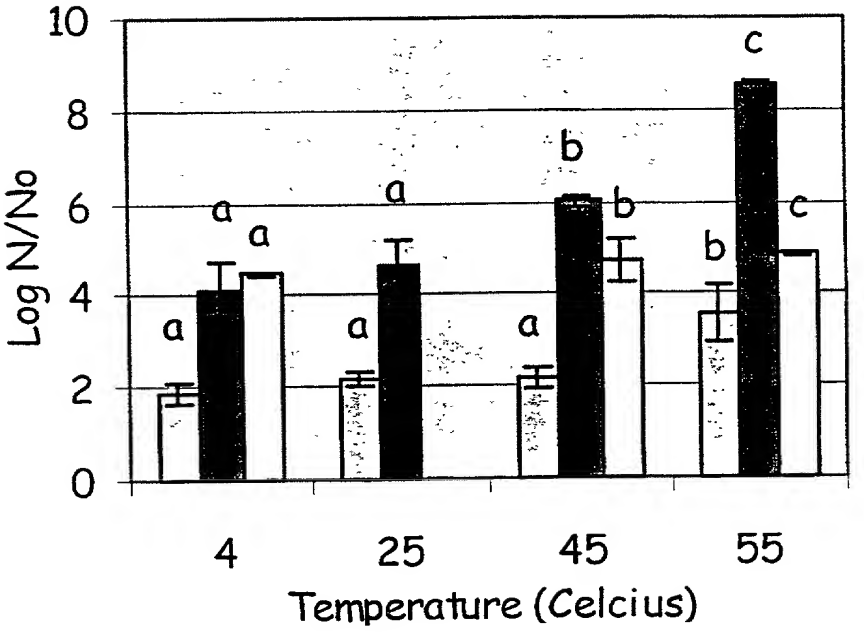
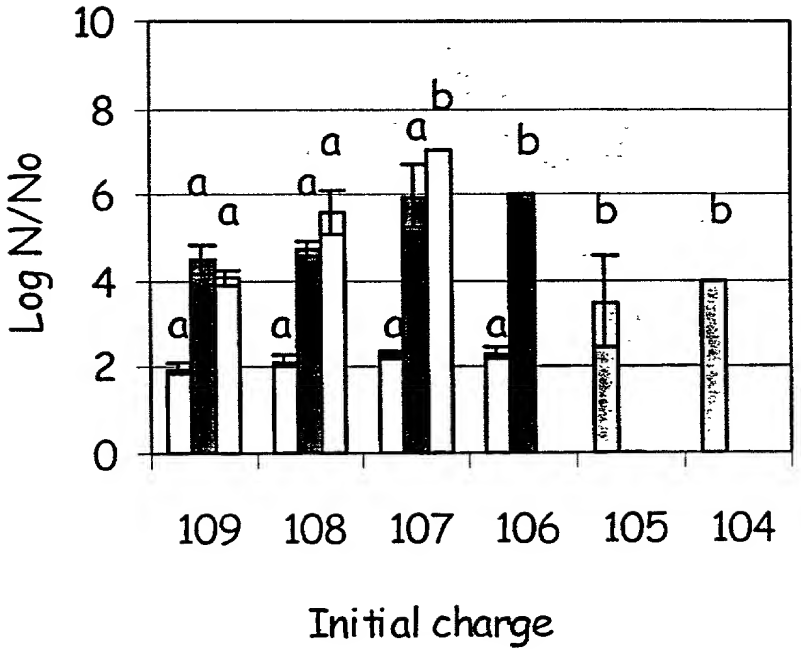


Fig. 2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a
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b
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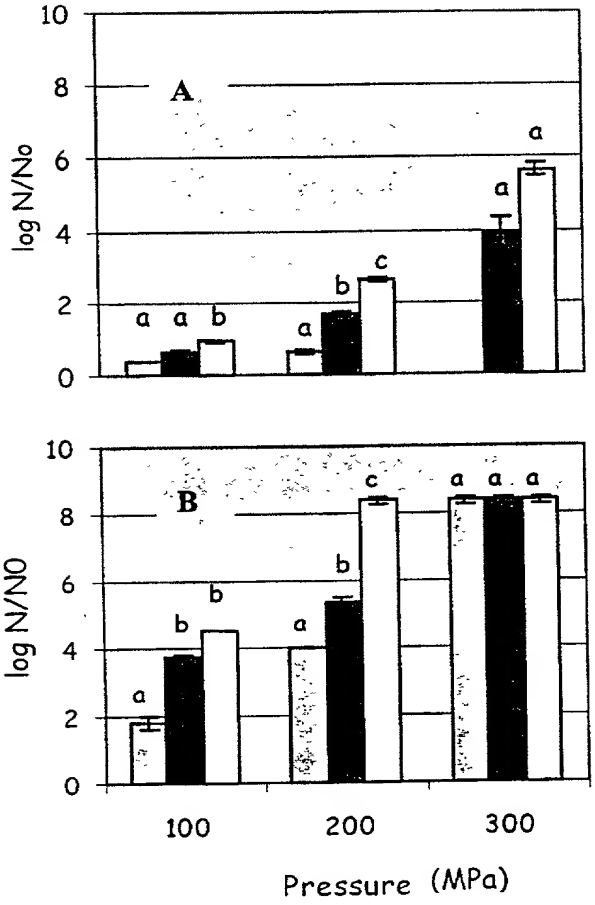
b
b
109
108
107
106
105
104
Initial charge

Fig. 3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log N/NO
10
8
6
4
2
0
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a b c
a a a
a b b
a b c
a a a
100
200
300
Pressure (MPa)

Fig. 4

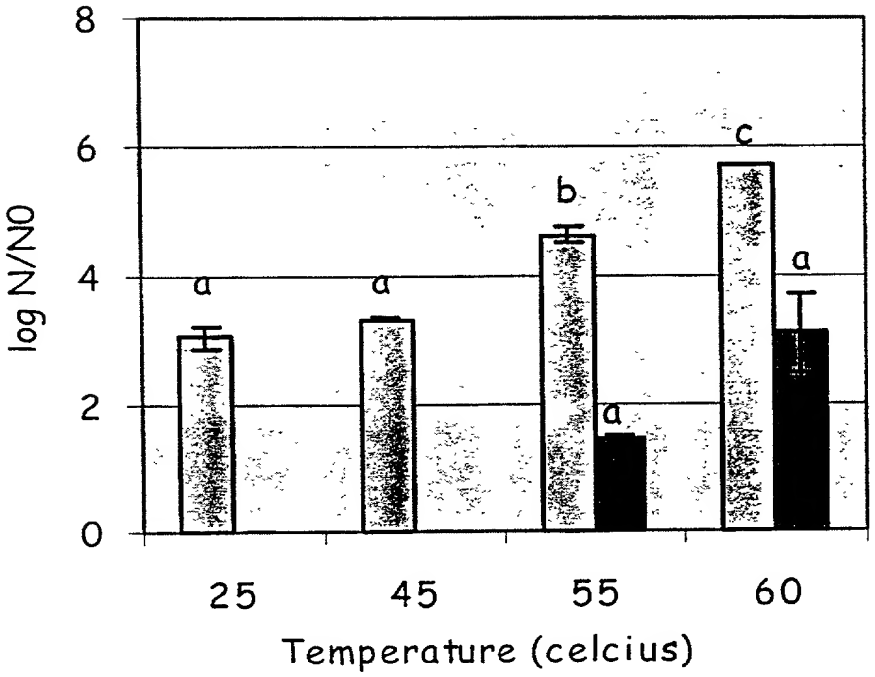

Fig. 5

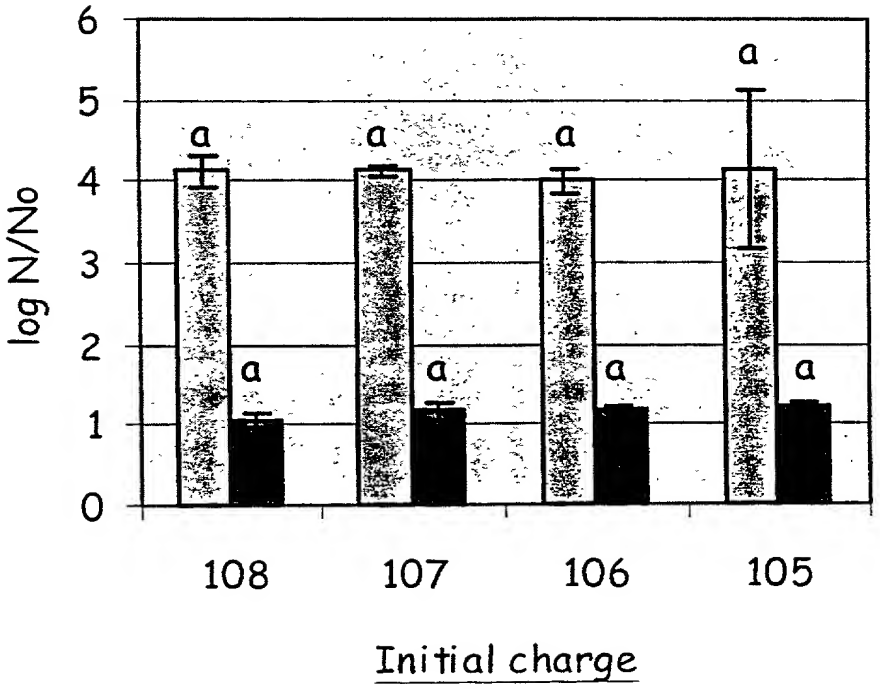

Fig. 6

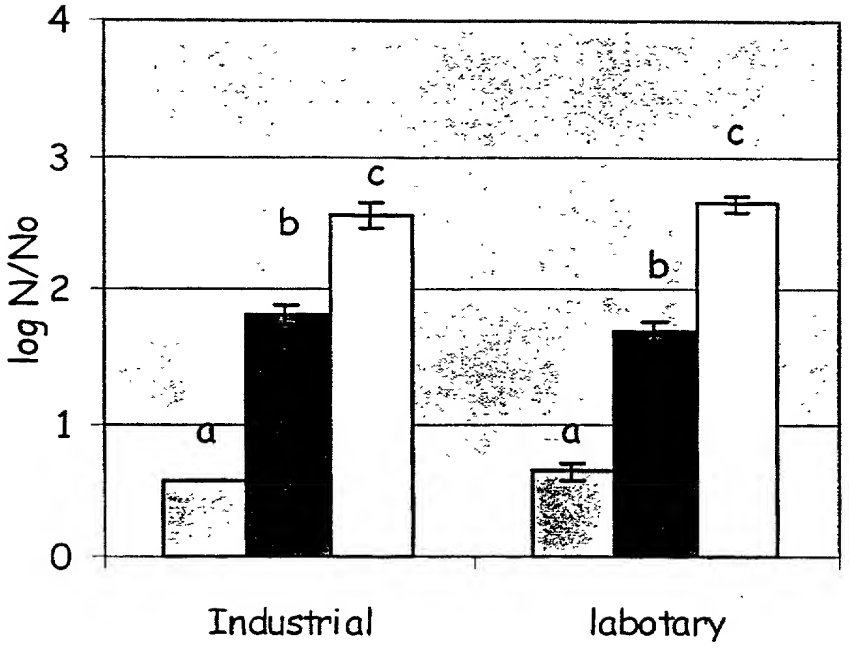

Fig. 7

216324US0PCT

Declaration, Power of Attorney and Petition

WE (I) the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

INACTIVATION OF FOOD SPOILAGE AND PATHOGENIC MICROORGANISMS BY DYNAMIC HIGH PRESSURE

the specification of which

☐ is attached hereto.

☒ was filed on 27 NOVEMBER 2001 ✓ As

Application Serial No. 09/926,622 ✓

and amended on _____.

☒ was filed as PCT international application

Number PCT/CA00/00621 ✓

on 25 MAY 2000 ✓,

and was amended under PCT Article 19

on _____ (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s)

Application No.	Country	Day/Month/Year	Priority Claimed
			☐ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No

We (I) hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

60/136,780 ✓	28 MAY 1999 ✓
(Application Number)	(Filing Date)
(Application Number)	(Filing Date)

We (I) hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or under § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Serial No.	Filing Date	Status (pending, patented, abandoned)
PCT/CA00/00621 ✓	25 MAY 2000 ✓	

And we (I) hereby appoint the following registered practitioner(s):

22850

as our (my) attorneys, with full powers of substitution and revocation, to prosecute this application and to transact all business in the Patent Office connected therewith; and we (I) hereby request that all correspondence regarding this application be sent to

22850

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1-00 Paul PAQUIN
NAME OF FIRST SOLE INVENTOR

Residence: 7593 Des Grebes, Charny, Quebec G6X 2C2, Canada CAX

Signature of Inventor

Citizen of: CANADA ✓

Mailing Address: SAME AS ABOVE

16/1/2002
Date

2-0D Jocelyne GIASSON
NAME OF SECOND JOINT INVENTOR

Jocelyne Giasson
Signature of Inventor

30-01-02
Date

Residence: 4540 St-Laurent, Levis,
Quebec G6V 3V9, Canada CAX

Citizen of: CANADA ✓

Mailing Address: SAME AS ABOVE

3-0D Jean-Francois VACHON
NAME OF THIRD JOINT INVENTOR

Jean Francois Vachon
Signature of Inventor

30-01-02
Date

Residence: 651 Genest, Thetford Mines,
Quebec G6G 6S2, Canada CAX.

Citizen of: CANADA ✓

Mailing Address: SAME AS ABOVE

4-0D Ismail ELISS
NAME OF FOURTH JOINT INVENTOR

Signature of Inventor

18/01/2002
Date

Residence: 3316 Lambert Closse, Ste-Foy,
Quebec G1W 2S4, Canada CAX.

Citizen of: CANADA ✓

Mailing Address: SAME AS ABOVE

NAME OF FIFTH JOINT INVENTOR

Signature of Inventor

Date

Residence:

Citizen of:

Mailing Address: